Preparation of Samples for Gas Chromatography/Mass Spectrometry Analysis of Phthalate and Adipate Esters in Plasma and Beverages by Steam Distillation and Extraction

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Phthalate and adipate esters are present in relatively large amounts in the environment, resulting in their large blank values at analysis and making precise analysis difficult. We developed a highly sensitive analytical method for phthalate and adipate esters in plasma and beverages by lowering the blank values that interfere with analysis. The method uses a closed distillation cleanup system in which steam distillation and extraction are performed simultaneously. The recoveries from beverages and plasma were both satisfactory, ranging from 90.2 to 118.3%, relative standard deviation (RSD) = 2.8–5.3%, and 96.2–134.4%, RSD = 2.2–6.5%, respectively. The detection limits of dibutyl phthalate and di-2-ethyl hexyl phthalate were 5 ng/mL, and those of diethyl phthalate, butyl benzyl phthalate, and di-2-ethyl hexyl adipate were 10 ng/mL in rabbit plasma and beverages.

Plastic products made of materials such as vinyl chloride resins are widely used not only as household goods but also as medical supplies, including blood tubing for artificial dialysis, kits for blood transfusion (tubes and bags), and catheters. Inexpensive plasticizers, such as phthalate and adipate esters, have been added to vinyl chloride resins to improve their quality and workability; however, toxicological properties of several phthalates that have eluted from plastic containers into food and have been taken into the body have been described in the literature (1–3). Thus, a highly sensitive analytical method is needed for such plasticizers in food and in biological samples. However, because the processes of extracting the distillate with an appropriate organic solvent and concentrating the extract involve a risk of contamination by plasticizers, the use of steam distillation for analysis of plasticizers in food and in biological samples is difficult. Accordingly, it has been suggested that a distillator in which steam distillation and extraction are performed in a closed system may be satisfactory for preparation of such samples (11–14).

Experimental

Reagents

(a) Solvents.—Hexane, acetone, and methanol were phthalate analysis grade (Kanto Kagaku, Tokyo, Japan). Toluene was dioxin analysis grade (Kanto Kagaku).

(b) Chemicals.—Anhydrous sodium sulfate was pesticide analysis grade (Wako Pure Chemical Industries Ltd., Osaka, Japan).

(c) Water.—Milli Q water (Millipore, Bedford, MA).

(d) Reference materials.—Diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), di-2-ethyl hexyl phthalate (DEHP), di-2-ethyl hexyl adipate (DEHA), DEP-d4, DBP-d4, BBP-d4, and DEHP-d4 were environmental analysis grade (Kanto Kagaku). DEHA-d8 was environmental analysis grade (Hayashi Pure Chemical Industries, Osaka, Japan).

(e) Standard solutions.—All were prepared with hexane so that each of the standard solutions contained one of the phthalate and adipate esters at 1000 ng/mL, and were diluted when used.
(f) Internal standard solutions.—All were prepared with methanol so that each of the solutions contained one of the internal reference materials at 1000 ng/mL, and were diluted when used.

(g) Standard solutions for calibration curves.—All were prepared so that each of the standard solutions of phthalate and adipate acid esters contained one of the internal reference materials at 100 ng/mL.

Instrumentation

(a) Gas chromatography/mass spectrometry (GC/MS) system.—HP5896 GC with Series HP5971 mass selective detector and 7673 autosampler (Hewlett-Packard, Palo Alto, CA).

(b) Coolant.—Eyela Cool Ace CA-1110 (Tokyo Rikakiki Co. Ltd., Tokyo, Japan).

Apparatus

(a) Essential oil distillation cleanup system.—See Figure 1.

(b) Glassware.—All were washed with acetone, followed by heating for at least 4 h in a dryer at 200°C.

Table 1. Accuracy data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DEHA</th>
<th>DEP</th>
<th>DBP</th>
<th>BBP</th>
<th>DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity (ng/mL)</td>
<td>6–1000</td>
<td>6–1000</td>
<td>6–1000</td>
<td>6–1000</td>
<td>6–1000</td>
</tr>
<tr>
<td>Correlation (r)</td>
<td>0.998</td>
<td>0.997</td>
<td>0.995</td>
<td>0.998</td>
<td>0.997</td>
</tr>
<tr>
<td>Detection limit (ng/mL)</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Retention time RSD, %</td>
<td>0.1</td>
<td>0.08</td>
<td>0.1</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>Peak area RSD, %</td>
<td>0.3</td>
<td>0.5</td>
<td>1.3</td>
<td>0.9</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Figure 1. Distillation cleanup system.

Figure 2. SIM chromatograms of standard solution.

Figure 3. Removal of plasticizers from water.

Conditions for Gas Chromatography/Mass Spectrometry Analysis

The column used in the experiment was a DB-5MS (J&W Scientific, Folsom, CA) 0.25 mm id x 30 m, film thickness 0.25 μm. The carrier gas was He (0.9 mL/min). For analysis of plasma, initial column temperature was 150°C, maintained for 3 min, and then raised to 280°C at elevation rate of 10°C/min. For analysis of beverages, initial column temperature was 50°C, raised to 280°C at elevation rate of 10°C/min. Inlet temperature was 280°C, and detector temperature was 280°C. A 1 μL amount of each sample was injected. Selected-ion monitoring (SIM) was performed at m/z 149 for DEP, DBP, BBP, and DEHP; at m/z 153 for DEP-d4, DBP-d4, BBP-d4, and DEHP-d4; at m/z 129 for DEHA; and at m/z 137 for DEHA-d8.

For GC/MS conditions, we used different initial column temperatures between 50 and 150°C for beverage and plasma, respectively, because coexisting substance from beverage sometimes affected chromatographic resolutions when the sample was analyzed at initial column temperature of 150°C.

Preparation of Samples

After addition of water (500 mL) into a round-bottomed flask (1000 mL), the flask was connected to the distillator (Figure 1), and a small amount of water and 4 mL toluene...
were placed in the distillator, to which a Dimroth condenser was attached. Before sample preparation, the water was distilled for 8 h to remove plasticizer contaminants in the apparatus. During distillation, toluene in the distillator was replaced every 2 h. After 8 h, the toluene and water in the distillator were discarded and a sample (either plasma or a beverage, 4 mL) and methanol solution (100 mL) containing one of the internal standards (1000 ng/mL) were added into the flask. A 1 mL amount of fresh toluene was accurately measured and fed into the distillator, to which a Dimroth condenser was attached. After 4 h of distillation, the extracted layer of toluene was taken into a small test tube, to which a small amount of anhydrous sodium sulfate was added for dehydration, and a sample was obtained for GC/MS analysis.

**Determination**

The samples were prepared to have specific concentrations, the peak areas were calculated by GC/MS, and the calibration curves were obtained according to the internal standard method of using deuterated compounds.

**Results and Discussion**

The present study describes an analytical method for DEP, DBP, BBP, DEHP, and DEHA, which are frequently found in environmental and food samples.

### Table 2. Recovery test of plasticizers from distilled water

<table>
<thead>
<tr>
<th>Added, ng/mL</th>
<th>DEHA</th>
<th>DEP</th>
<th>DBP</th>
<th>BBP</th>
<th>DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without I.S.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>83.9 (13.5)</td>
<td>45.6 (10.8)</td>
<td>67.4 (30.0)</td>
<td>46.2 (33.0)</td>
<td>110.5 (40.3)</td>
</tr>
<tr>
<td>With I.S.</td>
<td>102.5 (1.9)</td>
<td>93.7 (2.8)</td>
<td>94.4 (4.3)</td>
<td>95.8 (1.8)</td>
<td>100.3 (2.8)</td>
</tr>
<tr>
<td>100</td>
<td>98.3 (0.5)</td>
<td>94.5 (1.1)</td>
<td>101.3 (2.9)</td>
<td>96.8 (1.3)</td>
<td>99.8 (2.2)</td>
</tr>
</tbody>
</table>

* n = 4. GC conditions used for beverages.

* I.S. = internal standard.

### Determination by GC/MS

The retention time relative standard deviation (RSD) values, peak area RSD values, linearity, and detection limit [signal-to-noise (S/N) ratio = 3] of each sample are shown in Table 1; SIM chromatograms are shown in Figure 2. Because the RSD of the retention time of each sample was ≤0.1%, the following experiment was conducted under the conditions for GC/MS analysis described above.

**Removal of Plasticizers from Water**

In the present method, a sample and water are heated and circulated, and the volatile component is collected in toluene. Thus, if a plasticizer is present in the water, it will be concentrated in toluene, resulting in a high background value. Such plasticizers must be removed from the water to be used before sample preparation. Milli Q water without further purification was heated and circulated for 4 h, and toluene, which was analyzed by GC/MS, was found to contain DBP at 110–455 ng/mL (295 ng/mL average) and DEHP at 120–280 ng/mL (200 ng/mL average). However, as shown in Figure 3, these plasticizers in the Milli Q water were completely removed after 8 h of distillation. Although DBP and DEHP in the water can be removed as described above, other plasticizers may still be present. Accordingly, 5 types of plasticizers (DEHA, DEP, DBP, BBP, and DEHP, 1000 ng each), were fed into the Milli Q water after 8 h of distillation to study their removal. None of these plasticizers were detected after 8 h of distillation. Therefore, it was confirmed that 8 h of distillation removes all of these plasticizers.

### Table 3. Recovery test of plasticizers from rabbit plasma

<table>
<thead>
<tr>
<th>Added, ng/mL</th>
<th>DEHA</th>
<th>DEP</th>
<th>DBP</th>
<th>BBP</th>
<th>DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>99.0 (2.2)</td>
<td>103.0 (6.5)</td>
<td>103.0 (4.2)</td>
<td>134.4 (6.3)</td>
<td>96.2 (5.2)</td>
</tr>
<tr>
<td>100</td>
<td>98.3 (2.2)</td>
<td>107.4 (5.3)</td>
<td>104.2 (4.2)</td>
<td>112.5 (4.8)</td>
<td>112.5 (4.8)</td>
</tr>
<tr>
<td>500</td>
<td>100.8 (0.9)</td>
<td>107.4 (1.8)</td>
<td>101.2 (1.0)</td>
<td>108.9 (3.2)</td>
<td>102.6 (1.3)</td>
</tr>
</tbody>
</table>

* n = 7; concentration of internal standard, 100 ng/mL.

### Table 4. Recovery test of plasticizers from beverage

<table>
<thead>
<tr>
<th>Added, ng/mL</th>
<th>DEHA</th>
<th>DEP</th>
<th>DBP</th>
<th>BBP</th>
<th>DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>103.2 (3.9)</td>
<td>90.2 (2.8)</td>
<td>102.4 (4.7)</td>
<td>118.3 (5.3)</td>
<td>101.3 (3.8)</td>
</tr>
<tr>
<td>100</td>
<td>97.3 (2.9)</td>
<td>93.5 (1.5)</td>
<td>100.3 (2.9)</td>
<td>105.8 (4.2)</td>
<td>100.8 (2.2)</td>
</tr>
</tbody>
</table>

* n = 4; concentration of internal standard, 100 ng/mL.
Recovery from Water

Each of the plasticizers with a concentration of 500 or 100 ng/mL was added to 4 mL purified water (Milli Q water with further purification as described above) to perform the recovery test. The recoveries after 4 h of distillation are shown in Table 2. According to the absolute calibration curve method, DEHP and DEHA exhibited satisfactory recoveries, whereas DBP exhibited a slightly low recovery of 67.4%, and DEP and BBP showed very low recoveries of 45.6 and 46.2%, respectively. Also, the RSDs varied greatly, ranging from 10.8 to 40.3%. According to the distillation method, the target compound in the flask boils at 100 °C when heated with water, vaporizes with water, and then cools in the coolant provided at the upper part of the distillator (Figure 1). The water obtained and the target compound drip into the extract solvent placed in the middle of the distillator, and the target compound is then extracted.

The low recoveries of DEP and BBP were attributed to the low extraction efficiencies of these compounds in the extract solvent with their relatively high water-solubility. To improve recoveries, distillation was performed for more than 4 h. However, recoveries were not significantly higher than those obtained after 4 h of distillation, which suggests that the concentration of a plasticizer has reached equilibrium between the extract solvent and the dripped solution after 4 h of distillation. However, addition of stable isotopes (100 ng each) serving as internal standard materials into the flask before distillation improved the recoveries to 94.5–101.3% (RSD ≤ 2.9%) at a concentration of 500 ng/mL, and 93.7–102.5% (RSD ≤ 4.3%) at 100 ng/mL. Thus, we selected a distillation time of 4 h.

Recovery from Plasma

Whole blood was collected from a rabbit (Japanese white, male, 3000–3500 g) by venipuncture into glass tubes with heparin by using a glass syringe; this was followed by centrifugation for 20 min at 3000 rpm. The plasma obtained was subjected to a recovery test. Table 3 shows that the average recoveries of each of the plasticizers in 7 measurements ranged from 100.8 to 108.9% (RSD = 0.9–3.2%) at a concentration of 500 ng/mL, and from 98.3 to 112.5% (RSD = 2.2–5.3%) at 100 ng/mL. Even at a lower concentration of 25 ng/mL, recoveries were as good as 96.2–134.4% (RSD = 2.2–6.5%). The detection limits of DBP and DEHP were 5 ng/mL, and those of DEP, BBP, and DEHA were 10 ng/mL. Therefore, this distillation method would be satisfactory for analysis of plasticizers in biological samples. We analyzed female human plasma. No DEHA, DEB, or BBP was detected; however, DBP and DEHP were detected, with a DBP concentration of 30 ng/mL and a trace of DEHP.

Recovery from Beverages

Each of the plasticizers with a concentration of 100 or 25 ng/mL was added to 4 mL of a beverage for the recovery test (Table 4). The recoveries were satisfactory, ranging from 93.5 to 105.8% (RSD = 1.5–4.2%) at a concentration of 100 ng/mL, and from 90.2 to 118.3% (RSD = 2.8–5.3%) at 25 ng/mL. With this distillation method, the detection limits of DBP and DEHP in samples were 5 ng/mL, and those of DEP, BBP, and DEHA were 10 ng/mL.

Because the results suggested that this distillation method is applicable to food available on the market, we applied the method to commercially available samples. Eight commercial beverages in plastic containers were subjected to the analysis. The results are shown in Table 5, and their typical profile (beverage G) is shown in Figure 4. No DEHA, DEB, or BBP was detected in any of the samples; however, DBP and DEHP were detected in all of the samples, with the DBP concentration ranging from 5.80 to 105.3 ng/mL and the DEHP concentration ranging from 18.8 to 36.3 ng/mL.

Conclusions

Operational blank values were reduced by preparing Milli Q water, followed by heating and circulating the prepared water in a distillator for at least 8 h, with extraction and removal of any phthalate contaminants with toluene. Standard solutions of each plasticizer were added to beverages and
plasma so that the concentrations for the recovery tests would be 25 and 100 ng/mL. The recoveries from beverages and plasma were both satisfactory, ranging from 90.2 to 118.3% (RSD = 2.8–5.3%) and 96.2–134.4% (RSD = 2.2–6.5%), respectively.

The results showed that this distillation method permits a highly sensitive analysis, minimizing contamination by external factors because the operation is performed in a closed system, and that the method can be satisfactorily applied to analysis of plasma and beverage samples.

Acknowledgment

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References